Plant Cell Reports (1997) 16: 632–636

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Production of diosgenin by hairy root cultures of *Trigonella foenum-graecum* L.

Received: 22 April 1996 / Revision received: 27 June 1996 / Accepted 19 August 1996

Abstract Hairy root cultures of *Trigonella foenum-graecum* L. were established with *Agrobacterium rhizogenes* strain A_4. The hairy roots produce diosgenin, an important spirostanol for the semi-synthesis of steroid hormones. Fourteen different liquid media were investigated. The fastest growth was obtained in McCown's woody plant (WP) medium supplemented with 3% sucrose; the highest diosgenin content was observed in half-strength WP medium with 1% sucrose (0.040% dry weight), which represents almost twice the amount detected in the 8-month-old non-transformed roots (0.024%). A time-course study in WP liquid media supplemented with 3% sucrose was undertaken. In these conditions, 17 µg diosgenin/g fresh weight were produced. The influence of cholesterol, medium pH and chitosan on diosgenin production was tested. The addition of 40 mg/l chitosan elevated the diosgenin content to three times that found in non-elicited hairy roots.

Key words *Trigonella foenum-graecum* · Hairy roots · Diosgenin · Elicitor

Abbreviations *MS* Murashige and Skoog (1962) medium · *WP* McCown's woody plant medium

Introduction

As part of our investigations on secondary metabolites produced by transformed root cultures (Christen et al. 1989; Gränicher et al. 1992), we report here on the establishment of hairy roots of *Trigonella foenum-graecum* L. (Fabaceae) and their ability to biosynthesize diosgenin (Fig. 1). In vitro production of diosgenin in this species has already been reported (Khanna and Jain 1973; Brain and Lockwood 1976; Rodriguez-Mendiola et al. 1991) but optimization of the hairy root culture conditions has not been previously investigated. Diosgenin and phytosterols are very important as raw materials for the semi-synthesis of steroid hormones. Formerly, diosgenin was extracted from the tubers of Mexican and Asian species of yam. In 1976, however, state control of the relevant wild yam cultures of Mexico coupled with an ever-increasing demand for raw steroid led the industry to look for an alternative source of diosgenin (Coppen 1979). Fazli and Hardman (1968) suggested that the culture of fenugreek could be commercially developed so that it could be used both as a foodstuff and as a source of diosgenin.

In this report, we first describe the effects of *T. foenum-graecum* L. transformation by *Agrobacterium rhizogenes* strain A_4. Another objective was to investigate the influence of medium pH, cholesterol and chitosan concentrations on growth and diosgenin production.

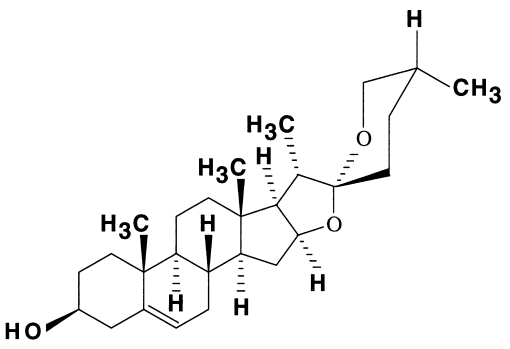

Fig. 1 Diosgenin

Communicated by P. Matile

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Materials and methods

Bacterial strain

A. rhizogenes strain A_4, an agropine-type strain ($pRiA_4$), was used in the present study. The bacteria were grown on solidified yeast mannitol broth medium (Hooykaas et al. 1977) and subcultured every month.

Plant material

Seeds were surface sterilized with a sodium hypochlorite solution (1.5% wt/vol available chlorine) supplemented with two drops Triton X-100 (Fluka) for 6 min and rinsed three times with sterile distilled water. They were left for germination on agar plates containing 0.5% sucrose at 25°C in the light with a daily 16-h photoperiod (light source: Osram-L-Fluora 77R, 700 lux). Plantlets were transferred onto solid half-strength McCown's woody plant (WP) medium (Lloyd and McCown 1980) +3% sucrose (1/2 WP 3) and maintained at 25°C in the light.

Establishment of hairy root cultures

Hairy roots were induced by direct infection of the stems of 2-week-old sterile plantlets with *A. rhizogenes* A_4. Approximately 4 weeks after infection, hairy roots were transferred onto solid 1/2 WP 3 medium containing 0.25 g/l cefotaxime and 1 g/l ampicillin (Sigma). After elimination of the bacteria, the hairy roots were inoculated into 1/2 WP 3 liquid medium (50 ml in 250-ml conical flasks), cultured in darkness at 25°C on a gyratory shaker (80 rpm) and subcultured at 4-week intervals. To prove transformation, the opines were extracted and identified by paper electrophoresis according to the method described by Petit et al. (1983).

Growth index

A growth index was established as: fresh weight after 35 days of culture/fresh weight of the inoculum.

Time-course study

Hairy roots (100 mg fresh weight) were inoculated into 50 ml liquid WP medium supplemented with 3% sucrose in 250-ml conical flasks and cultured in darkness at 25°C on a gyratory shaker at 80 rpm for 50 days. All media were hormone free and adjusted to pH 5.9 with 0.1 N NaOH before autoclaving.

Addition of cholesterol

Cholesterol (Sigma) was dissolved in ethanol and added to the medium (WP 3) after autoclaving (30, 50 and 70 mg/100 ml). Water-soluble cholesterol (Sigma; 10 mg/100 ml) was added to the medium prior to sterilization.

pH experiments

The pH of the medium was adjusted to 5.0–5.9 with 0.1 N NaOH before autoclaving.

Chitosan preparation

Chitosan (Sigma) was dissolved in 6% (w/v) acetic acid, centrifuged (10 500 *g*, 15 min) and precipitated by addition of 5 N NaOH. This procedure was repeated twice. The chitosan residue was washed three times with distilled water and freeze dried. Final chitosan concentrations in hairy root cultures were 0.5, 1, 2.5, 5, 10, 20, 30, 40, 50 and 60 mg/l. No chitosan was added to the control.

Extraction of diosgenin

The contents of three flasks of each culture were harvested, and fresh and dry weight, after lyophilization, were determined individually. The hairy roots were powdered and extracted essentially as described by Sauvaire and Baccou (1978). Each sample (200 mg dry weight) was extracted and hydrolyzed by refluxing for 5 h with 1 M sulfuric acid in 70% isopropanol. The extract was adsorbed on an Extrelut (Merck, Germany) column and diosgenin was eluted with *n*-hexane. The organic solvent was evaporated to dryness. Root tissues of normal 8-month-old plants grown in the field were extracted as described above. After filtering off cell debris, the liquid media were lyophilized and extracted using the same procedure.

Identification and quantification of diosgenin

Diosgenin was identified by high-performance liquid chromatography coupled with mass spectrometry and comparison with reference material. The selected procedure was based on the formation of the 3-benzoyl derivative essentially as described by Higgins (1976): 2 ml of dried pyridine (Merck, Germany) and 0.1 ml of benzoyl chloride (Fluka) were added to the dried extract. The sample was heated at 80°C for 30 min then, after addition of 2 ml of methanol, heated again at 100°C for 30 min. After cooling to room temperature, 10 ml of dichloromethane was added, followed by 20 ml of water and 2 ml of concentrated hydrochloric acid. The organic phase was washed successively with water, with a saturated sodium carbonate solution, and finally twice with water. The dichloromethane layer was evaporated to dryness and the residue was dissolved in chloroform (HPLC grade) before injection into the HPLC column. Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone, Fluka) was used as internal standard. Twenty microliters of filtered extract-internal standard mixture was injected. Diosgenin benzoate concentrations were determined by comparison with an external standard curve over the range 25–250 μg/ml.

HPLC analyses were performed using a Waters 600E apparatus on a Nucleosil 100-5 C-18 column [250×4 mm internal diameter (ID)] (Macherey-Nagel) fitted with a Nucleosil 120-5 C-18 guard column (11×4 mm ID). The isocratic solvent system was acetonitrile-water 92:8 (vol/vol) for 60 min with UV detection at 230 nm. The flow rate was set at 2 ml/min and the column temperature was maintained at room temperature.

Results and discussion

Optimization of the culture medium

Root growth and diosgenin content were examined in hairy roots cultured for 35 days in WP, Murashige and Skoog (1962) (MS) and Gamborg (1970) B5 media supplemented with 1–9% sucrose and compared with 8-month-old non-transformed plants grown in the field (Fig. 2). The fastest growth (606 mg) was obtained in WP supplemented with 3% sucrose (growth index: 80). The hairy roots cultured in WP (half and full strength) with a low sucrose concentration showed a higher diosgenin content. Increasing the strength of the WP medium did not significantly affect the root growth but was unfavorable for the diosgenin content. This phenomenon is particularly important with media containing 5, 7 and 9% sucrose. WP liquid medium re-

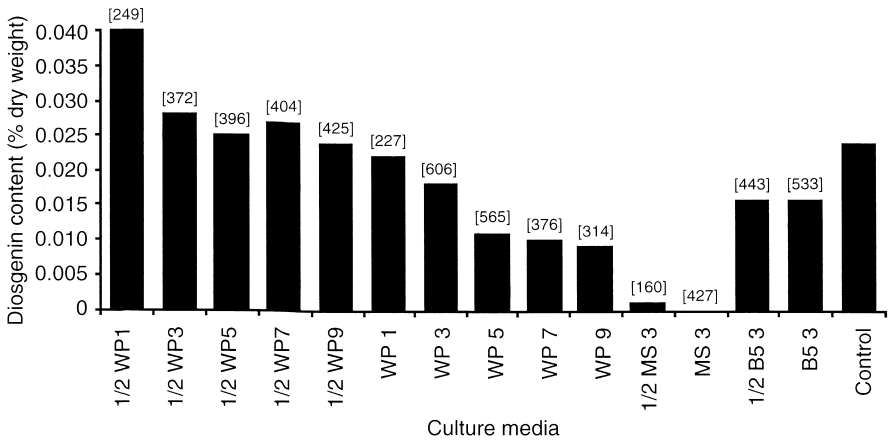

Fig. 2 Diosgenin content of hairy roots cultured in 14 different liquid culture media for 35 days. Non-transformed plants (*Control*) were cultured in the field for 8 months. *Numbers in parentheses* indicate the dry weight (mg)

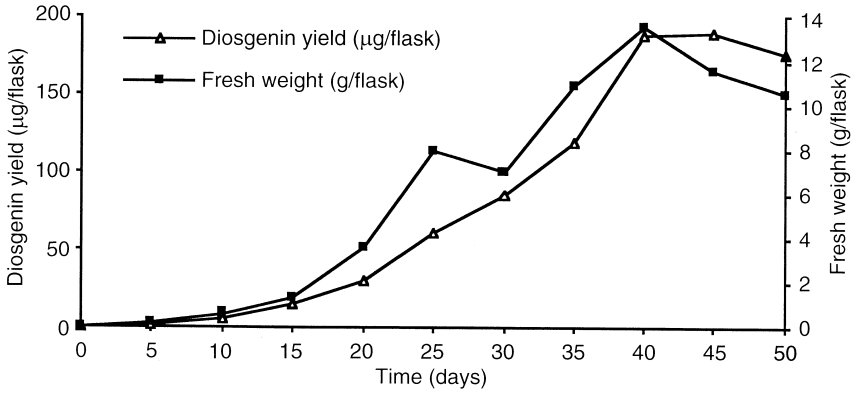

Fig. 3 Time-course study of growth and diosgenin yield in *Trigonella foenum-graecum* hairy roots cultured for 50 days in WP 3 medium

duced to half concentration and supplemented with 1% sucrose gave the highest diosgenin content (0.040% dry weight). This is about twice the amount detected in the control roots (0.024% dry weight).

With a growth index of only 17, half-strength MS medium supplemented with 3% sucrose was less favorable for both growth and diosgenin content. In MS 3 medium, diosgenin was not detectable (<0.4 μg/ml of culture medium).

Time-course study

In a further experiment, the time-course of growth and diosgenin production in WP 3 liquid medium was investigated (Fig. 3). At 5-day intervals, three flasks were harvested and root fresh weight and diosgenin production were measured. Over a 50-day culture period, the fresh weight of the hairy roots increased from an original inoculum of about 100 mg to 13.6 g (growth index: 136). The diosgenin content appeared to be closely related to the root growth and reached 0.028% dry weight after 50 days, representing a mean accumulation rate of 5.6 μg/g per day. Diosgenin production increased relatively slowly during the first 20 days, followed by a rapid rise for the next 25 days to reach a value of about 190 μg/flask after 45 days. Afterwards, both the fresh weight and the diosgenin yield decreased. The medium was also examined for the presence of diosgenin. During the 50 days of culture, no diosgenin was detected in the culture medium suggesting that all diosgenin was retained within the tissues.

Addition of cholesterol

Diosgenin is produced via the mevalonic acid pathway, cholesterol being a key intermediate in its biosynthesis. Khanna et al. (1975) reported a positive effect of cholesterol addition on diosgenin production in *T. foenum-graecum* suspension cultures. In our first experiment, three concentrations of cholesterol (30, 50 and 70 mg/100 ml) were added to the WP 3 liquid medium and tested for 35 days. The cholesterol was dissolved in a minimum volume of alcohol. A control medium (no cholesterol added) was used to determine whether alcohol had an inhibitory effect: growth was never observed. Cholesterol, after evaporation of ethanol, precipitated in the medium, so to avoid the influence of alcohol and the precipitation of cholesterol, water-soluble cholesterol encapsulated into the cavity of methyl β-cyclodextrin was tested at a concentration of 10 mg/100 ml. Preliminary experiments showed that

Table 1 Effect of initial pH of the WP 3 medium on growth and diosgenin content

pH before autoclaving	pH after autoclaving	pH after 35 days	Biomass dry weight (mg)	Diosgenin content (% dry weight)
5	4.8	4.8	730	0.042
5.5	5.2	5.2	710	0.037
5.9	5.7	5.6	605	0.018

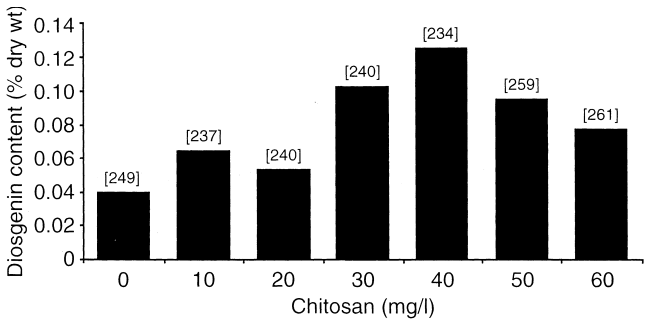

Fig. 4 Diosgenin content of hairy roots cultured for 35 days in 1/2 WP 1 containing various concentrations of chitosan. *Numbers in parentheses* indicate the dry weight (mg)

methyl β-cyclodextrin alone (30 mg/100 ml) did not influence hairy root growth. Of the three batches tested, this treatment leads to a large decrease in biomass, from 606 mg in the control to 265, 281 and 220 mg (dry weight), respectively, while the diosgenin yield (5 µg/flask) was only about 5% of the control level (108 µg/flask). These results were not in line with those reported by Khanna et al. (1975) for cell suspension cultures of *T. foenum-graecum*.

Influence of pH

Little is known about the effect of pH on growth and secondary metabolite production in hairy root cultures. We investigated the effects of pH on growth and diosgenin content in hairy roots cultured in WP liquid medium containing 3% sucrose (Table 1). The pH of the WP 3 medium was adjusted to 5.0, 5.5 and 5.9 with 0.1 N NaOH prior to autoclaving resulting in pH values of 4.8, 5.2 and 5.7 after autoclaving, respectively. The transformed roots were allowed to grow for 35 days and harvested. The biomass was not strongly affected and the roots were able to grow at all pH values. However, growth was slightly inhibited at pH 5.9. The effect of pH was more pronounced on the diosgenin content. Maximum diosgenin formation (0.042% dry weight) was recorded at pH 5.0. This is about three times the percentage obtained at pH 5.9. It is noteworthy that in all cases, the pH remained remarkably stable between the beginning and the end of the experiment.

Addition of chitosan

Chitosan is a polymer of β-1,4-D-glucosamine, which is obtained by alkaline hydrolysis of shellfish chitin. It is well known as an inducer of plant secondary metabolites (Funk and Brodelius 1990). In a preliminary experiment (data not shown), the addition of chitosan (0.5–40 mg/l) to WP liquid medium supplemented with 3% sucrose, the best medium for the hairy root growth, had no significant effect. Relatively little difference was observed between the control (no addition of chitosan) and the WP liquid medium treated with the elicitor. Therefore, various concentrations (10–60 mg/l) were added to half-strength WP liquid medium supplemented with 1% sucrose, the best medium for diosgenin yield, and the transformed roots were cultured for 35 days (Fig. 4). The addition of chitosan had little effect on the hairy root growth in comparison with the control. On the other hand, the diosgenin content was significantly increased in all cases by the addition of chitosan. Medium supplemented with 40 mg/l chitosan showed the highest diosgenin content (0.125% dry weight). This is three times the amount detected in the control roots (0.040% dry weight) and five times the amount detected in the non-transformed roots. These results clearly indicated that chitosan could greatly improve the diosgenin content of hairy root cultures.

The present study demonstrates that diosgenin production by hairy root cultures of *T. foenum-graecum* L. transformed with *A. rhizogenes* strain A_4 can serve as a very interesting system for studying the biosynthesis of this compound. Transformed roots cultured in half-strength WP medium supplemented with 1% sucrose for 35 days have the highest diosgenin content (0.040% dry weight). This is about twice the amount detected in the roots of 8-month-old non-transformed plants grown in the field. Cholesterol, a metabolic precursor, did not exert any positive influence on the hairy root growth and diosgenin production. On the other hand, a small decrease in the initial pH of the medium (5.0 instead of the usual 5.9) stimulated both growth and diosgenin content. The most remarkable effect was achieved by the addition of chitosan to 1/2 WP 1 medium. In this case, the diosgenin content increased up to five times (0.125% dry weight) the levels found in non-transformed roots.

Acknowledgements The authors would like to thank Dr. J. L. Wolfender (University of Lausanne) for running the LC-MS analysis.

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